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Rationally designed chemokine-based toxin targeting the viral G protein-coupled receptor US28 potently inhibits cytomegalovirus infection in vivo

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The use of receptor–ligand interactions to direct toxins to kill diseased cells selectively has shown considerable promise for treatment of a number of cancers and, more recently, autoimmune disease. Here we move the fusion toxin protein (FTP) technology beyond cancer/autoimmune therapeutics to target the human viral pathogen, human cytomegalovirus (HCMV), on the basis of its expression of the 7TM G protein-coupled chemokine receptor US28. The virus origin of US28 provides an exceptional chemokine-binding profile with high selectivity and improved binding for the CX3C chemokine, CX₃CL1. Moreover, US28 is constitutively internalizing by nature, providing highly effective FTP delivery. We designed a synthetic CX₃CL1 variant engineered to have ultra-high affinity for US28 and greater specificity for US28 than the natural sole receptor for CX₃CL1, CX₃CR1, and we fused the synthetic variant with the cytotoxic domain of *Pseudomonas Exotoxin A*. This novel strategy of a rationally designed FTP provided unparalleled anti-HCMV efficacy and potency in vitro and in vivo.

HCMV | 7TM GPCR | protein engineering | antiviral drug | chemokine

High-affinity receptor–ligand interactions using targeting antibodies or ligands have been exploited to direct toxins as fusion toxin protein (FTP) molecules to target a number of neoplastic diseases. An FTP between the monoclonal antibody trastuzumab linked to DM1 (ado-trastuzumab emtansine, Kadcyla) showed substantial response rates in the treatment of breast cancer and was approved for this purpose by the US Food and Drug Administration in 2012 (1). Similarly, an FTP comprising an anti-CD22 monoclonal antibody variable fragment region (engineered for increased ligand-binding affinity) fused to the cytotoxic domain of *Pseudomonas aeruginosa* Exotoxin A (PE) (2) is currently in phase III trials for relapsed and refractory hairy cell leukemia (3, 4). However, most FTP-based approaches targeting cancer have met with more limited success (5, 6). In addition to pharmacological challenges and poor cell-targeting specificity, a primary cause of failure is insufficient antigen internalization to achieve direct killing of tumor cells (7–10).

The potential for FTP-based approaches to target infectious disease based on the expression of pathogen-encoded high-affinity receptors by infected cells has not been explored. Human cytomegalovirus (HCMV) is a clinically important opportunistic viral pathogen in individuals with immature or compromised immune function (11). Ganciclovir (GCV, Cytovene) remains the gold-standard and first-line treatment for HCMV in most clinical settings (12). Similar to all approved anti-HCMV drug therapies used for prophylactic, preemptive, or curative treatment of HCMV, GCV targets the viral DNA replication machinery (13). Although effective, the current approved drugs fail

in preventing HCMV disease in particular settings, e.g., lung-, heart–lung-, pancreas-, and allogeneic hematopoietic stem cell transplantation and immunodeficiency (14, 15). Moreover, these drugs have treatment-limiting side effects, including serious nephro-, neuro-, and hematologic toxicity, and are susceptible to the frequent development of drug-resistant strains, with single mutations commonly conferring resistance to multiple drugs across the class (16–19). Together, these limitations support the value of developing new drug treatments with novel mechanisms of action that could be used to complement existing therapies and to treat disease refractory to DNA polymerase inhibitors because of resistance.

The molecular interactions between human and virus-encoded receptors and their cognate chemokines are becoming well understood (20–23). We decided to explore the potential of a novel HCMV antiviral strategy based on targeting HCMV-infected

Significance

All drugs currently used for the clinical treatment of human cytomegalovirus (HCMV) infection are associated with considerable adverse side effects and with the development of drug resistance that results in therapy failure. Here we describe a novel, rationally designed fusion toxin protein (FTP)-based strategy to target HCMV on the basis of its virally expressed G protein-coupled receptor (US28) and cognate chemokine ligand. Viral G protein-coupled receptors are expressed by a number of other clinically important viruses. We suggest that FTP-based molecules targeting virally expressed 7TM receptors may represent a new class of drugs amenable for development against complex viral pathogens.

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cells through their expression of a virus-encoded seven-transmembrane (7TM) chemokine receptor, US28. A number of pharmacological and cellular properties of US28 suggest that this viral G protein-coupled receptor might be suited for targeting HCMV with a FTP-based strategy (Fig. 1*A* and *B*). First, US28 binds a broad spectrum of chemokines as part of its immune-evasive function through chemokine scavenging. Importantly, US28 shows high selectivity and enhanced binding for the CX₃C chemokine CX₃CL1 (24, 25). In contrast to most other human chemokines, CX₃CL1 consists of a chemokine domain, a mucin stalk, and a transmembrane domain (Fig. 1*C*) providing membrane anchorage of CX₃CL1 (26). The chemokine domain of CX₃CL1 has high affinity for US28 both after cleavage and when CX₃CL1 is attached to the mucin-like stalk extrusion from the chemokine domain (24, 25). Moreover, CX₃CL1 binds only a single human chemokine receptor, CX₃CR1, thus decreasing the potential for unwanted off-target effects of a CX₃CL1-based FTP strategy. Second, the majority of US28 is localized within endosomes, away from the cell surface (27). This distribution is a result of rapid, constitutive, ligand-independent receptor internalization

(28) and is a feature suited for efficient intracellular delivery of FTP molecules. Based on the molecular characteristics of US28 and its defined ligand-binding profile, we designed an FTP consisting of the chemokine domain of CX₃CL1 and domains of PE (Fig. 1*C–E*). The structural characteristics of CX₃CL1 suggest that the protein can sustain high-affinity binding to US28 when the C-terminally attached mucin-like stalk is replaced by other proteins, e.g., the PE domains associated with translocation (domain II) and cytotoxicity (domains Ib and III). PE is a very potent toxin capable of killing cells via its adenosine diphosphate-ribosylation domain that modifies elongation factor 2, leading to the arrest of protein synthesis and the initiation of apoptosis (29, 30).

The aim of the current study was to explore an FTP-based approach using an engineered chemokine fused to a toxin to target a human viral pathogen, HCMV, on the basis of its expression of the constitutively internalizing 7TM receptor, US28 (Fig. 1*B*).

Results

CX₃CL1-FTP Has Increased Binding Affinity for HCMV US28 Compared with CX₃CR1

First, to determine whether a toxin could be targeted to HCMV-infected cells via cell surface-expressed US28, we created a fusion protein, CX₃CL1-FTP (Fig. 1*E*). The CD91 receptor binding of PE (domain I) was removed to limit entry to only US28-expressing cells. Consistent with CX₃CL1's high affinity for US28, which is unaltered whether the chemokine domain is alone or is attached to the mucin stalk (24, 25), the replacement of the mucin-like stalk with three PE domains to create the CX₃CL1-FTP construct did not prevent binding to US28 (Fig. 1*F*). Importantly, CX₃CL1-FTP maintained high affinity for US28 with a less than fourfold decrease in affinity compared with the WT CX₃CL1 chemokine [0.19 nM (log IC₅₀ −9.7 ± 0.52) and 0.67 nM (log IC₅₀ −9.2 ± 0.08), respectively, using ¹²⁵I-CCL2 as the radioligand] (Fig. 1*F*). In contrast, CX₃CL1-FTP's affinity for CX₃CR1 was 145-fold less than that of CX₃CL1 [IC₅₀ = 53 nM (log IC₅₀ −7.3 ± 0.15) and 0.37 nM (log IC₅₀ −9.4 ± 0.12), respectively, using ¹²⁵I-CX₃CL1 as the radioligand] (Fig. 1*G*). Together, these results show that fusion of CX₃CL1 to PE domains results in a recombinant FTP molecule (CX₃CL1-FTP) that has a selectivity profile that favors binding to US28 with 80-fold higher affinity for US28 than for CX₃CR1 (Fig. 1*F* and *G*).

CX₃CL1-FTP Preferentially Kills Cells Expressing US28

To assess the capacity of CX₃CL1-FTP to deliver the chemokine-coupled PE toxin payload intracellularly, we measured *in vitro* cell-killing activity in cells expressing either US28 or CX₃CR1 under the control of a tetracycline-inducible promoter. As shown in Fig. 1*H*, CX₃CL1-FTP effectively killed US28-expressing cells, with 60% cell killing at 0.1 nM [IC₅₀ = 0.03 nM (log IC₅₀ −10.5 ± 0.37)]. Consistent with its decreased affinity for CX₃CR1, 0.1 nM CX₃CL1-FTP caused a lower degree of cell killing (10%) in CX₃CR1-expressing cells but retained high potency [IC₅₀ = 0.39 nM (log IC₅₀ −9.4 ± 0.48)]. In contrast, cells with no receptor expression (noninduced cells) showed high viability even at 1 nM CX₃CL1-FTP. The observed differences in maximum killing of cells expressing US28 compared with cells expressing CX₃CR1 (70% versus 40%) may be caused by differences in receptor cellular distribution profiles and/or recycling properties (27). Together, these results show that US28-expressing cells are killed efficiently by CX₃CL1-FTP. The reduced killing of cells expressing CX₃CR1 compared with cells expressing US28 is consistent with CX₃CL1-FTP's higher affinity for US28 than for CX₃CR1 (Fig. 1*F* and *G*).

CX₃CL1-FTP Kills HCMV-Infected Cells With Potency Surpassing That of GCV

To test the antiviral efficacy and potency of CX₃CL1-FTP within the context of infection, CX₃CL1-FTP was added to HCMV (Towne strain)-infected human foreskin fibroblasts (HFFs). In single-dose experiments, CX₃CL1-FTP completely inhibited the

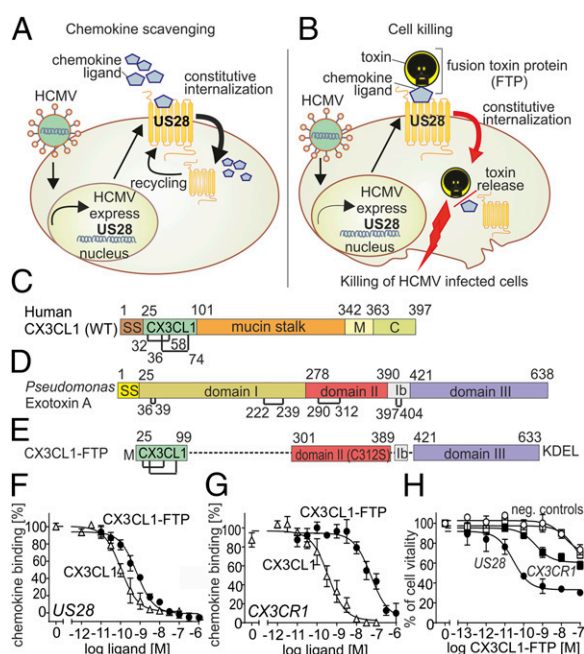


Fig. 1. Mode of action, design, binding, and killing properties of the CX₃CL1-FTP. (*A*) Immune evasion strategy by US28-mediated chemokine binding and constitutive internalization. (*B*) Cell killing by targeting this immune evasion pathway using a US28-targeting FTP consisting of a CX₃CL1-derived chemokine and cytotoxic domain of PE. After US28 binding and internalization, proteolytic cleavage releases the toxin from CX₃CL1, resulting in cell death. (*C*) Human CX₃CL1: signal sequence (SS); CX₃CL1, chemokine domain; mucin stalk; membrane spanning domain (M); and cytoplasmic domain (C). (*D*) *Pseudomonas* Exotoxin A (PE): signal sequence (SS); CD91 receptor-binding domain (I); translocation domain (II); domain with unknown function (Ib); enzymatic cytotoxic domain (III). (*E*) CX₃CL1-FTP with the CX₃CL1 chemokine motif and a modified PE consisting of domain II (amino acids 301–389) and part of domain Ib and domain III (amino acids 406–633), but without domain I; replacement of cysteine 312 by serine (C312S), and optimized KDEL sequence. (*F* and *G*) Binding of CX₃CL1-FTP (black circles) and CX₃CL1 (open triangles) on HEK 293 cells induced to express US28 (*F*) and CX₃CR1 (*G*). (*H*) Cell killing of CX₃CL1-FTP on tetracycline-induced HEK 293 cells expressing US28 (black circles) and CX₃CR1 (black squares) together with noninduced cells with no receptor expression (negative controls: open circles and squares, respectively). For the cell-killing experiments the data are normalized to the maximum number of living cells. Error bars in *F–H* indicate SEM for three or four independent biological replicates.

release of virus particles, with a potency surpassing by 10^4 -fold that of the current first-line HCMV drug, GCV [$IC_{50} = 1.9$ nM (log $IC_{50} -8.7 \pm 0.08$) and $18 \mu\text{M}$ (log $IC_{50} -4.7 \pm 0.11$), respectively] (Fig. 2A). In repeated-dose experiments in HCMV-infected cells, cultures treated with 0.3 nM (log concentration -9.5) or higher doses of CX₃CL1-FTP developed a fully confluent cell monolayer by 8 days postinfection indicating complete control of virus replication (Fig. 2B). Consistent with its lower potency, $30 \mu\text{M}$ (log concentration -4.5) GCV was required to achieve a similar antiviral effect. HFFs are in principle susceptible to CX₃CL1-FTP, because detectable CX₃CR1 expression levels have been published in these cells (31). However, because CX₃CL1-FTP's affinity for CX₃CR1 is 80-fold lower than its affinity for US28, a much higher concentration of the FTP would be required to kill CX₃CR1-expressing cells (Fig. 1F and G). Collectively, these results show that, in the context of HCMV infection, CX₃CL1-FTP has high potency for killing US28-expressing cells.

The Antiviral Activity of CX₃CL1-FTP Is Caused by Direct Killing of HCMV-Infected Cells. To investigate whether the antiviral activity of CX₃CL1-FTP was a consequence of direct and selective killing of HCMV-infected cells, we studied the inhibition of viral replication in human fetal lung fibroblasts (MRC-5) infected with the HCMV Toledo_{LUC} strain that contains a luciferase reporter gene (32). In this modified strain, luciferase activity can be monitored as a direct measure of viral replication (33). Compared with the laboratory Towne strain, Toledo also is regarded as having characteristics more similar to clinical HCMV isolates (34). In single-dose experiments, CX₃CL1-FTP inhibited luciferase activity efficiently, with a potency 10^6 -fold higher than that

of GCV [$IC_{50} = 1.7$ nM (log $IC_{50} -8.8 \pm 0.18$) and >2 mM (log IC_{50} higher than -2.7), respectively] (Fig. 2C), independent of the multiplicity of infection (MOI) (Fig. S1). In repeated-dose experiments, treatment of infected cells with 1 nM CX₃CL1-FTP inhibited luciferase activity below the detection level (Fig. 2D). This robust level of viral control could not be obtained with GCV, where an increase in luciferase activity began at 3 days postinfection, even when using a 10^5 -fold higher concentration (0.1 mM) (Fig. 2D). CX₃CL1-FTP also was compared with another currently used anti-HCMV therapeutic, Kiovig (human normal Ig administered i.v.) and again showed superior efficacy and viral control in both single- and repeated-dose experiments (Fig. S2).

The Antiviral Activity of CX₃CL1-FTP Is Effective Against GCV-Resistant HCMV Clinical Isolates. To assess further the clinical potential of CX₃CL1-FTP, antiviral activity was evaluated using a GCV-resistant clinical isolate, HCMV-SV2. This strain remained highly sensitive to CX₃CL1-FTP but, as expected, was relatively unaffected by GCV treatment, with the potency of CX₃CL1-FTP being 4×10^5 -fold greater than the potency of GCV in single-dose experiments (Fig. 2E). In repeated-dose experiments, 1 nM [log concentration -9] CX₃CL1-FTP resulted in complete virus control after 6 days of treatment (Fig. 2F). Speed of action is an important feature of an antiviral drug, particularly in treating immune-compromised transplant patients for whom the time of treatment can determine the difference between successful organ engraftment or rejection (35). Together, our results show that CX₃CL1-FTP is a highly potent inhibitor of both laboratory and clinical strains of HCMV. Moreover, the virocidal mechanism (i.e., killing of infected cells) of CX₃CL1-FTP appears to be intrinsically faster acting, at least in vitro, than the virostatic mode of action of GCV.

CX₃CL1-FTP Resistance After Long-Term Treatment in Vitro. To investigate whether treatment with CX₃CL1-FTP could lead to resistance, long-term treatment was initiated. The results show that CX₃CL1-FTP is not in itself unresponsive to the development of resistance in vitro. A CX₃CL1-FTP-resistant HCMV TOWNE strain appeared after 3 weeks of selection under CX₃CL1-FTP. The causative mutation was a single G-to-A mutation at position 774 within the US28 gene, resulting in a premature stop codon at amino acid residue 258 in the third extracellular loop of US28 (Fig. S3A). This truncated variant of US28 (1-257) was expressed at the cell surface, although to a lower degree than US28 WT (Fig. S3B). However, competition binding experiments with radiolabeled CX₃CL1 and CCL2 revealed impaired chemokine binding for US28 (1-257), and chemotaxis analyses confirmed that the US28 (1-257) had lost its ability to bind chemokines, because it failed to adhere to and migrate on surfaces presenting FL-CX₃CL1 (Fig. S3C–F). Because US28-mediated chemokine scavenging and migration are believed to be critical for immune evasion (36, 37), an inability of US28 to bind chemokines likely would affect the fitness of F49A-FTP-resistant variants and possibly would impact their capacity to persist in vivo.

The Selectivity of Rationally Designed CX₃CL1 Molecules for US28 Is Superior to That of CX₃CR1. Because CX₃CL1-FTP also killed cells expressing the human chemokine receptor CX₃CR1 (albeit much less efficiently; Fig. 1H), we used a rational design strategy to enhance further the selectivity of the FTP molecule toward US28. Based on our knowledge of chemokine domains important for receptor recognition and action (21, 22), we designed and expressed 35 different CX₃CL1 variants (numbered 1 through 35) with mutations in the CX₃CL1 chemokine component and measured their affinity for US28 and CX₃CR1 (Fig. 3A, Fig. S1, and Table S1). Because CC-chemokines and CX₃CL1 are known to bind to US28 through distinct mechanisms (24, 25), we used two radioligands (^{125}I -CCL2

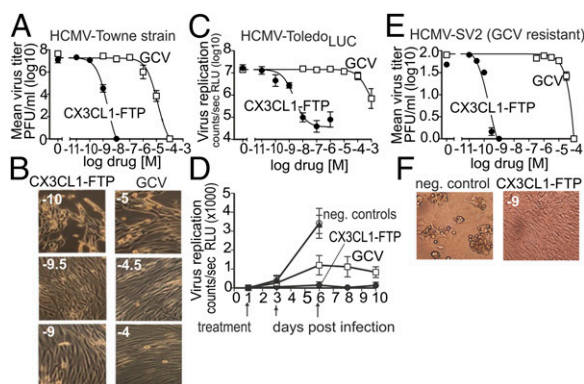


Fig. 2. Anti-HCMV activity of CX₃CL1-FTP. (A) Inhibition of virus particle release measured as pfu from HFFs infected with HCMV Towne at a concentration corresponding to one virus particle per 10 cells (MOI of 0.1) and treated with a single dose of CX₃CL1-FTP (black circles) or GCV (open squares). (B) HFFs 8 d after infection with HCMV Towne (MOI of 0.1). The cells were treated with CX₃CL1-FTP or GCV at the indicated concentrations (10^{-9} – 10^{-10} M (-9 , -9.5 , -10) and 10^{-4} – 10^{-5} M (-4 , -4.5 , -5), respectively) on days 1, 3, 5, and 8 postinfection. (C) Inhibition of virus replication measured by luciferase activity in human lung fibroblasts (MRC-5) infected with HCMV Toledo_{LUC} (MOI of 0.1) and treated with a single dose of CX₃CL1-FTP or GCV at the indicated concentrations. RLU, relative luminescence units. (D) Effect of repeated drug treatments on virus replication in MRC-5 cells infected with Toledo_{LUC} (MOI of 0.1). The cells were treated on days 1, 3, and 6 postinfection with CX₃CL1-FTP (10^{-9} M), GCV (10^{-4} M), or buffer (negative controls), and virus replication was measured by luminescence. (E) Inhibition of virus particle release from MRC-5 cells infected with the GCV-resistant HCMV-SV2 (MOI of 0.01) and treated twice (on days 2 and 3 after infection) with either CX₃CL1-FTP or GCV at the indicated concentrations. (F) MRC-5 cells 6 d after infection with the GCV-resistant HCMV-SV2 (MOI of 0.01) and treated twice (on days 2 and 3) with CX₃CL1-FTP (10^{-9} M) (Right) or buffer (Left). Error bars in A, C, D, and E indicate SEM for three independent biological replicates.

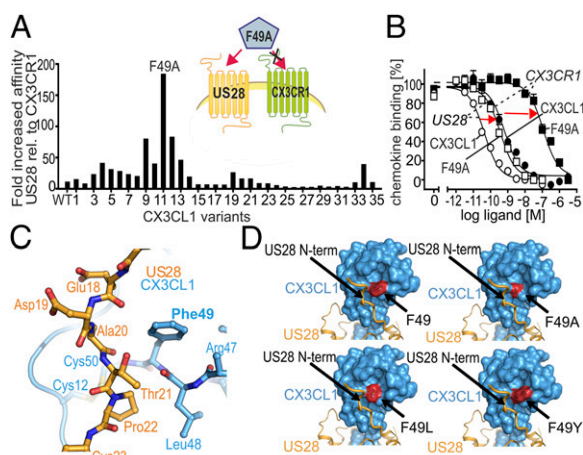


Fig. 3. Design, cell-killing, and antiviral activity of the US28-selective F49A-FTP. (A) Binding selectivity of WT and CX₃CL1 variants (1–35) determined as the fold improved affinity for US28 relative to CX₃CR1 (IC₅₀ values on CX₃CR1 over US28 using ¹²⁵I-CX₃CR1 as radioligand; Fig. S1). Variant 11 (F49A-CX₃CL1), with the highest US28 selectivity, was used for second-generation FTP, F49A-FTP. (B) Binding of WT CX₃CL1 (chemokine domain) and F49A-CX₃CL1 on HEK 293 cells induced to express US28 and CX₃CR1. Error bars indicate SEM of three independent biological replicates. (C) The interaction between US28 (orange) and CX₃CL1 (blue) is partially mediated by Phe⁴⁹ of CX₃CL1 (PDB ID code 4XT1). (D) Molecular models of the interaction between US28 (orange) and CX₃CL1 (blue), depicting mutagenesis of CX₃CL1 Phe⁴⁹ to alanine, leucine, or tyrosine (red).

and ¹²⁵I-CX₃CL1) to determine the affinity of US28 binding. Only ¹²⁵I-CX₃CL1 was necessary for the analysis of CX₃CR1 binding, because it is the only endogenous ligand for this receptor (Fig. S4 and Table S2). Five single-point mutants (variants 4, 6, and 9–11), one double mutant (variant 16), and three chimeric constructs (variants 31, 33, and 34) showed >100-fold reduction in their affinity for CX₃CR1, compared with WT CX₃CL1 (Fig. S4A). Three of these variants (16, 31, 33) exhibited a parallel reduction for US28 (Fig. S4B and C), whereas variants 4, 6, 9–11, and 34 maintained US28-binding capacity, resulting in 26- to 182-fold higher affinity for US28 than for CX₃CR1 (Fig. 3A).

Variant 11, with the highest US28 selectivity index (182-fold) (Fig. 3A and B and Table S2), was selected for characterization as a second-generation US28-targeting FTP-therapeutic (designated F49A-FTP). This decision was based on both the functional results and the structure of the US28-CX₃CL1 complex. The globular chemokine body interacts with the receptor N terminus and extracellular loops, and the chemokine N terminus enters the helical core of the receptor (23). The side chain of CX₃CL1 Phe⁴⁹ contacts Glu¹⁸ and Ala²⁰ of the US28 N terminus, and the F49A mutation results in an 18-fold decrease in affinity for US28. However, the F49A mutation disproportionately impacts CX₃CR1 binding, causing a 356-fold reduction in affinity (Fig. 3C and D and Table S1).

Consistent with its increased selectivity for US28, F49A-FTP had a highly selective cell-killing profile with ~10³-fold higher potency for US28-expressing cells than for CX₃CR1-expressing cells [IC₅₀ = 0.03 nM (log IC₅₀ -10.5 ± 0.24) and >30 nM (log IC₅₀ higher than -7.6), respectively] (Fig. 4A). CX₃CL1-FTP killed 10% of CX₃CR1-expressing cells at 0.1 nM (Fig. 1H), but F49A-FTP showed no cytotoxicity at this concentration; a 100-fold higher concentration was required to achieve a similar 10% decrease in viability (Fig. 4A).

F49A-FTP Controls HCMV Replication in Vitro and in Vivo with Greater Potency than GCV. F49A-FTP maintained high anti-HCMV efficacy in both single- and repeated-dose experiments using the

HCMV-Toledo_{LUC} strain (Fig. 4B and C). The potency in single-dose experiments was ~2 × 10⁴-fold higher than that of GCV [IC₅₀ = 10 nM (log IC₅₀ -8.0 ± 0.13) and 0.28 mM (log IC₅₀ -3.6 ± 0.22), respectively] (Fig. 4B), and the efficacy of repeated treatments with 1 nM F49A-FTP also was superior to that of 0.1 mM GCV (Fig. 4C).

In a final series of experiments, the efficacy of F49A-FTP was assessed in vivo in an SCID-hu mouse model, a state-of-the-art model that enables the replication of clinical HCMV isolates to be analyzed in vivo (38). SCID-hu mice were implanted with human fetal thymus/liver tissue under the kidney capsule. After engraftment the implants were infected with the HCMV Toledo_{LUC} strain by direct inoculation. Luciferase activity was serially monitored as an indicator of in vivo virus replication in real time (i.e., without killing the mice) (Fig. 4D and E). In total, implants in 17 SCID-hu mice were surgically inoculated with Toledo_{LUC}, and these mice were divided into the following experimental treatment groups: 0.4 mg/kg F49A-FTP (*n* = 5), 8 mg/kg GCV (*n* = 5), or mock treatment (*n* = 7). Five additional mock-treated SCID-hu mice were included as noninfected controls. The GCV dose was based on previous studies using a similar model and on the clinical recommendations of 5 mg·kg⁻¹·d⁻¹ in patients (33, 39). One mock-treated infected mouse died at day 12 postinfection, but no mice in any of the other treatment groups died during the course of the experiment. The lack of gross toxic effects in uninfected

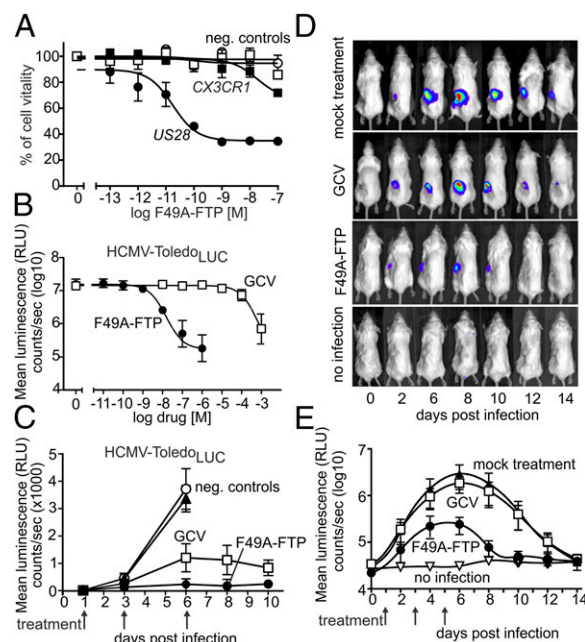


Fig. 4. Antiviral activity of the US28-selective F49A-FTP in vitro and in vivo. (A) Cell-killing activity of F49A-FTP in HEK 293 cells expressing US28 or CX₃CR1. (B) Inhibition of virus replication measured by luciferase activity of MRC-5 cells infected with Toledo_{LUC} (MOI of 0.1) and treated once with F49A-FTP (black circles) or GCV (open squares). (C) Effect on virus replication of repeated drug treatments of MRC-5 cells infected with Toledo_{LUC} (MOI of 0.1). Cells were treated on days 1, 3, and 5 after infection with F49A-FTP (10⁻⁹ M) (black circles), GCV (10⁻⁴ M) (open squares), or buffer (negative controls; open circle and black triangle). (D) Toledo_{LUC}-infected SCID-hu mice with thymus/liver implants. The animals were treated with 0.4 mg/kg F49A-FTP, 8 mg/kg GCV, or buffer (mock treatment) at 1, 3, and 5 d postinfection. (E) Quantification of the luciferase activity from D: F49A-FTP (black circles), GCV (open squares), buffer (mock-treated, black triangles), and noninfected mice (open triangles). For the cell-killing experiments, curves are normalized to maximum number of living cells. Error bars in A–C indicate SEM for three to eight independent biological replicates and error bars in E indicate SEM for five to seven animals per group.

mice indicates that there is no binding with subsequent receptor internalization of F49A-FTP to mouse CX₃CR1. Mock-treated HCMV-infected mice developed significant bioluminescence signals over the 14-day period of assessment, indicating highly productive HCMV replication. In contrast, F49A-FTP-treated mice showed only a transient, weak signal on days 4–6 post-infection which then dropped to background levels (Fig. 4 *D* and *E*). At peak luciferase activity (day 8), the bioluminescence signal of F49A-FTP-treated mice was 10-fold lower than that of GCV-treated mice. In summary, these results in the SCID-hu mouse model identify F49A-FTP as a highly effective inhibitor of virus replication *in vivo* that appears to be far superior to GCV.

Discussion

In the present study we show that an approach based on a rationally designed FTP molecule using a synthetic CX₃CL1 chemokine engineered for ultra-high affinity and selectivity for the US28 receptor can be used to develop a potent antiviral agent for HCMV. The efficacy of the derivative FTP-based antiviral drugs was superior to that of the anti-HCMV drugs GCV and Kiovig. To date, FTP-based strategies generally have been studied for the treatment of cancer and autoimmune disease. Our study also underscores the strength of rational drug design-based approaches both in the selection of appropriate receptor–ligand interactions and in further molecular refinement to reduce undesirable FTP-binding characteristics. This strategy resulted in the development of a second-generation FTP, F49A-FTP, that had a higher selectivity profile toward US28 and that controlled HCMV infection *in vivo* at levels surpassing GCV. The high efficacy of F49A-FTP demonstrates the potential of this molecule for further development as an anti-HCMV therapeutic drug. Moreover, the recently reported structure of the US28–CX₃CL1 complex providing the structural basis for chemokine recognition of US28 will further increase the possibility of developing this FTP into a drug with a superior therapeutic index. F49A-FTP's novel mechanism of action may increase treatment options of patients for whom GCV is not a possibility because of toxicity or the development of viral resistance to DNA polymerase inhibitors (18, 19). Combination therapy of F49A-FTP-based drugs with first-line agents also may allow lower levels to be used clinically, thereby reducing toxicity side effects. In contrast to virostatic DNA polymerase inhibitors such as GCV, FTPs inhibit HCMV by a virocidal mechanism of direct killing of infected cells before the release of virus progeny. This difference in the mechanism of virus control may be responsible for the more complete level of HCMV inhibition observed for F49A-FTP as compared with GCV. PE is known to induce apoptosis efficiently after delivery into the cell (30). Importantly, PE-mediated apoptosis is not blocked by the HCMV-encoded inhibitor of caspase 8 that inhibits programmed cell death pathways (40, 41). Many questions clearly remain, including the capacity for development of F49A-FTP resistance *in vivo*. Fusion to toxins of even greater biocidal activity may further enhance the FTP antiviral activity and thereby decrease the possibility of the development of drug resistance. Questions regarding immunogenicity associated with F49A-FTP also will need to be addressed, but thus far FTP-directed immune responses do not appear to affect the efficacy of FTPs substantially when used in cancer (42). Antibodies directed against a PE-based FTP in a mouse pulmonary fibrosis (autoimmunity disease) model were shown not to reduce the biological activity (43).

The crystal structure of the human CX₃CL1 chemokine domain bound to US28 serves as a model for viral chemokine 7TM-ligand complexes. Viral G protein-coupled receptors are expressed by a number of viruses in addition to HCMV, and some of these are anticipated to function in a manner similar to US28. Rationally designed FTP-based strategies targeting virus-expressed receptors may provide promising drug-target pairs not

only for anti-HCMV therapy but also for other viruses that express similar constitutively internalized receptors with known ligand-binding profiles.

Methods

Radioligand Competition Binding Assays. Stable inducible clones of US28/CX₃CR1-HEK293 cells were grown as previously reported (44). Briefly, the cells were seeded at 10,000 cells per well in poly-D-lysine (Invitrogen)-coated 96-well plates (Nunc). One day after seeding, US28 and CX₃CR1 expression was induced by tetracycline (3.6 ng/mL and 5 ng/mL, respectively) to obtain 5–10% specific binding. Competition binding studies were performed in triplicate as described previously (45). Briefly, 1 d after induction, cells were incubated for 3 h at 4 °C with 32 pM of the iodinated chemokine plus 6 μ L of the unlabeled ligand in 125 μ L buffer with 50 mM Hepes (Invitrogen) and 0.5% (wt/vol) BSA (Sigma). Afterward cells at 4 °C were washed twice with 200 μ L 50 mM Hepes buffer supplemented with 0.5% (wt/vol) BSA and 0.5 M NaCl. Data were collected using a Gamma-counter.

In Vitro Killing Activity. Stable inducible clones of US28/CX₃CR1-HEK293 cells were seeded at 5,500 cells per well in Poly-D-lysine-coated 48-well tissue-culture plates (Nunc) in 500 μ L DMEM (Invitrogen) per well and were grown at 37 °C. Receptor expression was induced 24 h after seeding using 0.25 μ g/mL (US28) and 0.125 μ g/mL (CX₃CR1) tetracycline. The different concentrations of the indicated FTP (0.1 μ M–0.1 pM) and buffer (mock treatment) were added 1 d after receptor induction in a final volume of 300 μ L DMEM and were incubated for 24 h at 37 °C. To estimate cell viability, the cells were incubated with AlamarBlue (Invitrogen) in PBS (5 mg/mL), 300 μ L per well, for 4 h at 37 °C. Data were collected using a Synergy HT plate reader.

Antiviral Activity. The antiviral effect of the immunotoxins was tested on the HCMV strains Towne and Toledo_{LUC} and on the GCV-resistant clinical isolate HCMV-SV2. The impact on the release of infectious virus particles from HFFs (ATCC CRL-1634) was measured using the HCMV Towne strain. For single-dose experiments HFFs (50,000 cells per well) were seeded in 24-well tissue-culture plates (Nunc) and were infected with HCMV Towne (ATCC VR-977) at an MOI of 0.1 (corresponding to one virus particle per 10 cells). The infection was allowed to proceed under exposure of a single dose of the respective FTP (1 μ M–10 pM) or GCV (Sigma) (0.1 mM–1 nM) until the infected untreated cells reached 100% cytopathic effect. Virus titers in supernatants were measured by plaque assay. For repeated-dose experiments HFFs (120,000 cells per well) were seeded in six-well tissue-culture plates and were infected with HCMV Towne at an MOI of 0.01. PBS (100 μ L) containing FTP, GCV, or control buffer was added every second day, and virus infection was documented by photographs.

The intracellular replication was measured using HCMV Toledo_{LUC} in MRC-5 human fetal lung fibroblasts (ATCC CCL-171). The recombinant HCMV Toledo_{LUC} virus was reconstituted from the HCMV BAC_{LUC} (26), and the expression of the luciferase gene from the viral genome was used as a quantitative measure of viral replication. For single-dose experiments MRC-5 cells (8,000 cells per well) were seeded in 96-well tissue-culture plates (Nunc) and were infected with Toledo_{LUC} at an MOI of 0.1. After the addition of a single dose of FTP (1 μ M–10 pM), GCV (Sigma) (0.1 mM–1 nM), or Kiovig (Baxter) (68 nM–21 μ M), the infection was allowed to proceed for 4 d; then the medium was removed, followed by the addition of 100 μ L PBS (MgCl₂; CaCl₂) and 100 μ L Britelite Plus reagent (PerkinElmer). Because Kiovig functions by virus neutralization, a preincubation step with Toledo_{LUC} was performed by incubating 100 pfu with different concentrations of Kiovig for 1 h at 37 °C before addition to wells. Luciferase activity was measured using a Synergy HT plate reader. For repeated-dose experiments, MRC-5 cells (60,000 cells per well) were seeded in 24-well tissue-culture plates (PerkinElmer) and were infected with Toledo_{LUC} at an MOI of 0.01. PBS (100 μ L) containing FTPs (1 nM), GCV (0.1 mM), or buffer was added every second day. Twenty-four hours postinfection the cells were given growth medium with D-Luciferin at a final concentration of 150 g/mL. The luciferase activity was measured on days 1, 3, 6, 8, and 10 postinfection using the Synergy HT plate reader.

Activity on a GCV-Resistant Clinical Isolate. MRC-5 cells (10,000 cells per well) were seeded in 24-well tissue-culture plates (Nunc) and were infected with the HCMV-SV2 strain (46) (kindly provided by Sebastian Voigt of the Robert Koch Institute, Berlin) at an MOI of 0.01. After virus adsorption, the virus inoculum was replaced with 1 mL of a methylcellulose overlay medium, and the cells were treated with different concentrations of CX₃CL1-FTP, GCV, or buffer (mock treatment) at 2 and 3 d postinfection. Cultures were incubated

for 9 d, and the plaques were evaluated visually. The progress of the HCMV-SV2 infection in MRC-5 cells (MOI of 0.1) after treatment with 1 nM of CX₃CL1-FTP and buffer (mock treatment) was visualized photographically at 6 d postinfection.

In Vivo Studies. Toledo_{LUC} was diluted to a titer of 2.5×10^6 pfus with PBS and was kept on ice until use. Thirty male homozygous C.B-17 SCID/SCID mice were surgically implanted with a human fetal thymus/liver sandwich-like structure under the kidney capsule, as previously described (33). Three months after implantation, the SCID-hu mice were anesthetized, and implants were surgically exposed and inoculated with 20 μ L Toledo_{LUC} (5×10^4 PFU). Luciferase activity was measured using an in vivo imaging system by injecting D-luciferin substrate i.p., as previously described (32, 33). The infected animals were treated by i.p. injection with buffer (mock), GCV (8 mg/kg), or F49A-FTP (0.4 mg/kg) on days 1, 3, and 5 postinfection. Bioluminescent measurements were performed every 48 h. All animals were included in the analysis, and animal groups were not blinded. Human fetal tissues were obtained from Advanced Bioscience Resources with informed consent according to local, state, and federal regulations. Animal work was done under the guidelines of the National Institutes of Health *Guide for the*

Care and Use of Laboratory Animals (47), and the protocol was approved by the Rutgers-New Jersey Medical School Institutional Animal Care and Use Committee.

Molecular Modeling. Mutations of CX₃CL1 Phe⁴⁹ were modeled on the 2.9-Å structure of CX₃CL1 bound to US28 [Protein Data Bank (PDB) ID code 4XT1] using PyMOL v1.7.4.0 (Schrodinger, LLC). For each mutation, the most probable rotamer that did not produce clashes was chosen. Structure figures were prepared with PyMOL.

Statistical Analysis. Data analyses were performed using Prism v6.0.1. Data are expressed as means \pm SEM.

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